Current Biology 24, 717–724, March 31, 2014 ©2014 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2014.02.037

Article

A Tree Ortholog of *APETALA1* Mediates Photoperiodic Control of Seasonal Growth

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Summary

Background: Photoperiodic control of development plays a key role in adaptation of plants to seasonal changes. A signaling module consisting of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) mediates in photoperiodic control of a variety of developmental transitions (e.g., flowering, tuberization, and seasonal growth cessation in trees). How this conserved CO/FT module can mediate in the photoperiodic control of diverse unrelated developmental programs is poorly understood.

Results: We show that *Like-AP1* (*LAP1*), a tree ortholog of *Arabidopsis* floral meristem identity gene *APETALA1* (*AP1*), mediates in photoperiodic control of seasonal growth cessation downstream of the CO/FT module in hybrid aspen. Using *LAP1* overexpressors and RNAi-suppressed transgenic trees, we demonstrate that short day (SD)-mediated downregulation of *LAP1* expression is required for growth cessation. In contrast with *AP1* targets in flowering, *LAP1* acts on *AINTEGUMENTA-like 1* transcription factor, which is implicated in SD-mediated growth cessation. Intriguingly, unlike *AP1* in *Arabidopsis*, ectopic expression of *LAP1* fails to induce early flowering in hybrid aspen trees.

Conclusions: These results indicate that *AP1* ortholog in trees has acquired a novel function in photoperiodic regulation of seasonal growth. Thus, photoperiodic signaling pathway may have diverged downstream of *AP1/LAP1* rather than the CO/FT module during evolution. Moreover, control of flowering by the CO/FT module can be uncoupled from its role in photoperiodic control of seasonal growth in trees. Thus, our findings can explain mechanistically how a conserved signaling module can mediate in the control of a highly diverse set of developmental transitions by a similar input signal, namely photoperiod.

Introduction

Photoperiod is a key environmental cue regulating developmental transitions as diverse as flowering time [1], tuberization [2], and seasonal growth cessation in trees [3]. A conserved signaling module consisting of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*), which was first identified in model plant *Arabidopsis* in the regulation of flowering time [4], plays a central role in photoperiodic control of development [1]. These observations have led to a question regarding the evolution of mechanism by which the CO/FT module can mediate in the control of diverse unrelated developmental programs by the photoperiodic signal. Analysis of transition to flowering, which is one of the best-characterized developmental programs, has shown that one of the main downstream targets of the CO/FT module is the floral meristem identity gene *APETALA1* (*AP1*) [5]. *AP1*, a MADS-box transcription factor [6], acts as a hub in controlling the expression of a range of downstream genes in order to induce floral transition and flower development in *Arabidopsis* [7]. In contrast with flowering, little is known about the downstream targets of the CO/FT module in photoperiodic control of other developmental programs. Therefore, identification of the downstream targets of the CO/FT module unrelated to flowering would shed light on how the photoperiodic pathway has evolved to control diverse developmental programs.

We have investigated photoperiodic control of seasonal growth in model experimental tree hybrid aspen. In trees growing in temperate and boreal forests, cessation of growth prior to the advent of winter is necessary in order to survive subsequent low temperatures [3, 8, 9]. The timing of growth cessation is a photoperiodically controlled process because these trees anticipate the advent of winter by measuring the reduction in day length [10]. When day length is shorter than a growth-permitting threshold (referred to as short days [SDs]), the formation and emergence of new leaves are terminated and the preformed leaf primordia are enclosed inside bud scales in a specialized bud structure [11]. The CO/FT module plays a central role in sensing SD signal, and the key event in the induction of growth cessation is the rapid downregulation of *FT2* expression after the perception of SDs [12]. Functional analysis of *FT* genes in the *Populus* species has shown that SD-induced downregulation of *FT2* expression is required for growth cessation. For example, hybrid aspen trees overexpressing either *FT1* or its paralog *FT2* fail to cease growth after SD treatment, whereas trees in which *FT* expression has been downregulated respond more quickly to SDs than wild-type (WT) counterparts [12–14].

Downstream of the CO/FT module, the *AINTEGUMENTA-like 1* (*AIL1*) transcription factor is the target of SDs [15, 16]. SD treatment results in the reduction of *AIL1* expression, which is essential for growth cessation [16]. Importantly, perturbing the SD perception (e.g., by *FT1* overexpression) prevents the downregulation of *AIL1* expression by SDs [16]. Because *AIL1* and related transcription factors (e.g., *ANT*) [17] are positive regulators of the expression of core cell-cycle genes (e.g., D type cyclins), the repression of *AIL1* expression by SDs results in the cessation of growth [16, 17].

Although *AIL1* acts downstream of the CO/FT module, the available data indicate that *FT* does not directly control *AIL1* expression [16]. Thus, it is not known how the perception of SDs via the CO/FT module results in the downregulation of *AIL1* expression and, hence, initiation of growth cessation [3]. Therefore, identification of the factors acting between CO/FT and *AIL1* would fill a major lacuna remaining in our understanding of the SD-mediated control of growth cessation. Furthermore, identification of these intermediate factors would provide insights into the divergence in photoperiodic signaling pathways downstream of the conserved CO/FT module.

We took a functional genomics approach in order to identify the components acting between the CO/FT module and *AIL1*

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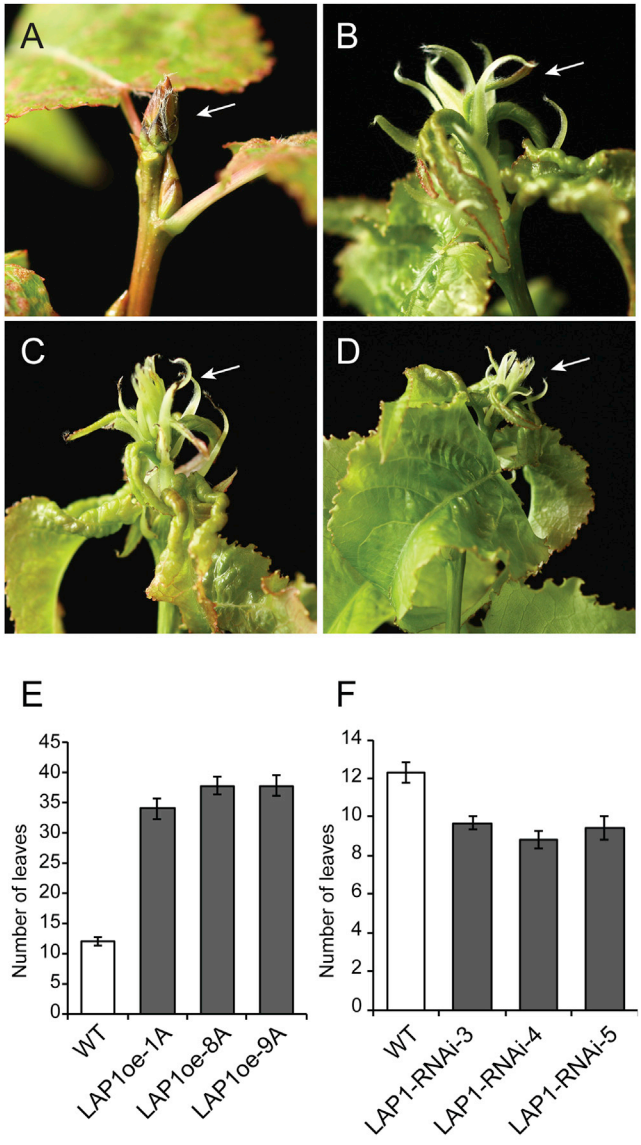

Figure 1. *LAP1* Overexpression Attenuates SD-Induced Growth Cessation

WT and LAP1oe plants were subjected to 10 weeks of SDs.

(A–D) The WT plants (A) ceased growth and formed buds (arrowed). In contrast, plants of three independent LAP1oe lines, designated 1A (B), 8A (C), and 9A (D), failed to cease growth and form apical bud (an actively growing apex is arrowed).

(E and F) Number of leaves formed until growth cessation after SD treatment was counted as a measure of SD response in the WT and three independent LAP1oe lines (1A, 8A, and 9A) (E) and in three independent LAP1-RNAi plants (3, 4, and 5) (F). Leaf numbers (y axis) indicate mean ± SE (n = 7).

See also Figure S1.

targeted by SDs. We screened transgenic hybrid aspen plants overexpressing *Populus* transcription factors for altered growth cessation response to SDs. This screen resulted in the identification of a MADS-box transcription factor called *Like-AP1* (*LAP1*), which is highly similar to the *Arabidopsis* floral meristem identity gene *AP1* [18]. Functional analysis of *LAP1* in hybrid aspen demonstrates that it is the target of SDs downstream of the CO/FT module and that *LAP1* mediates in the regulation of *AIL1* expression. Thus, *AP1*-like genes have acquired a novel function in trees in seasonal control of growth by a photoperiodic signal and suggest that the photoperiodic signaling pathway may have diverged downstream of *AP1/LAP1* rather than the upstream CO/FT module during evolution.

Results

Hybrid Aspen Plants with Altered *LAP1* Expression Have Attenuated Growth Cessation Response to SDs

We screened transgenic hybrid aspen lines overexpressing hybrid aspen transcription factors for altered growth cessation response to SDs. From this screen, we identified several independent lines overexpressing a transcription factor called *LAP1* (Figure S1A available online) in which the growth cessation response was severely attenuated (Figures 1A–1D). *LAP1* encodes a MADS-box transcription factor that is highly similar to the *Arabidopsis* floral meristem identity gene *AP1* [18] and its close relatives *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) [19] (Figures S2A and S2B). *Populus* genome also contains another gene, *PtAGL7*, that encodes a protein similar to *LAP1* (68% identity); however, PtAGL7 is more similar to FUL (Figure S2B), and its function is not currently known. WT plants ceased growing and formed apical buds (Figure 1A), whereas *LAP1*-overexpressing (LAP1oe) plants continued growing and did not form apical buds (Figures 1B–1D), even after 10 weeks of SDs.

Perception of SDs results in termination of leaf formation; thus, the number of leaves that emerges from commencement of exposure to SDs until growth cessation provides a sensitive measure of the SD response [20]. On average, WT plants had formed 12 new leaves after SD treatment until growth cessation (Figure 1E), but LAP1oe plants continued to grow and produce new leaves (34–37) until the end of the experiment (10 weeks of SDs) (Figure 1E). Thus, *LAP1* overexpression results in severe attenuation of SD-mediated growth cessation. We also generated transgenic hybrid aspen plants in which *LAP1* expression was downregulated (LAP1-RNAi) (Figure S1B). RNAi-mediated downregulation of *LAP1* expression in LAP1-RNAi lines significantly affected growth cessation in response to SDs because they produced fewer new leaves (8–9) during SD treatment than the WT controls (12–13) (Figure 1F).

LAP1 Is Functionally Similar to *Arabidopsis AP1*

Because LAP1 has high similarity at the amino acid level to *Arabidopsis* AP1, we investigated whether *LAP1* can function like *AP1*. For this purpose, we expressed *LAP1* cDNA in WT *Arabidopsis thaliana*. The results show that, like overexpression of *AP1* [21], *LAP1* expression in *Arabidopsis* leads to early flowering (Figures S3A–S3C). Whereas the WT produced 14–15 leaves, *LAP1* expressors produced 5–7 leaves before flowering in long days (Figure S3D). Furthermore, the primary shoot meristems of *LAP1* expressors were converted into compound terminal flowers, and secondary shoot meristems produced in axils of cauline and rosette leaves were transformed into solitary flowers, as observed in *Arabidopsis* following ectopic expression of *AP1* (Figures S3B and S3C). These results corroborate the hypothesis that hybrid aspen *LAP1* is functionally similar to *Arabidopsis AP1*.

LAP1 Is Expressed in the Apex and Downregulated by SD Treatment

Attenuation of the growth cessation response to SDs in LAP1oe and LAP1-RNAi plants prompted us to investigate

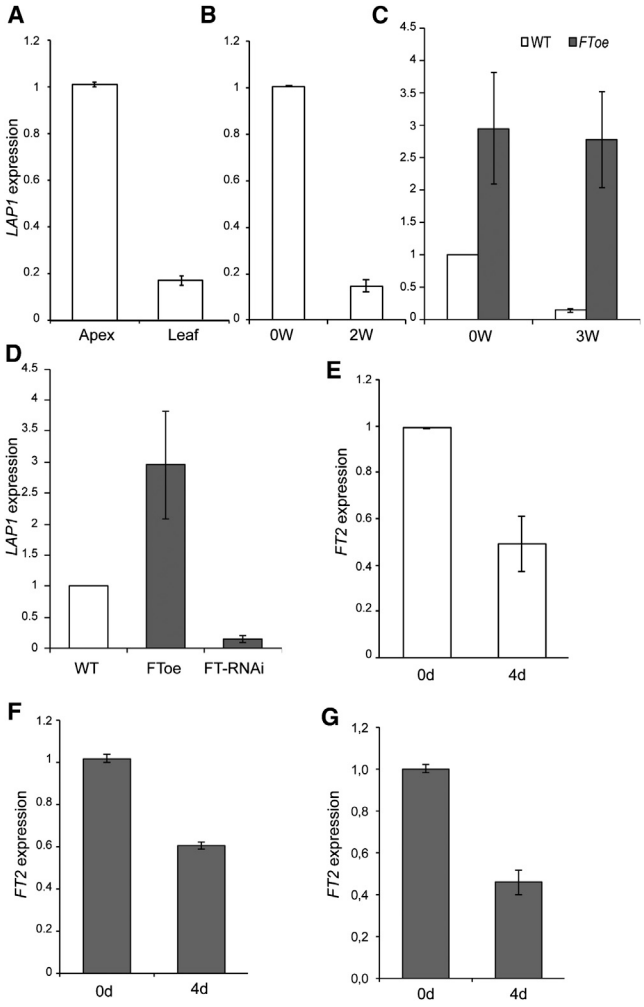

Figure 2. *LAP1* Is Expressed in the Apex and Is a Target of SDs Downstream of the CO/FT Module

(A) *LAP1* expression in the apex and leaves of 4-week-old WT plants in long days.

(B) *LAP1* expression in the apex of WT plants after 2 weeks of SDs.

(C) *LAP1* expression in the apex of WT and FToe (35S:FT1) plants after SDs.

(D) *LAP1* expression in the apices of WT, FToe, and FT-RNAi (35S:FT-RNAi) plants in LD conditions. *LAP1* transcript levels are (average of three biological replicates ± SE) relative to that of reference gene UBQ (y axis).

(E–G) *FT2* expression responds to SD signal in *LAP1*-overexpressing plants. *FT2* expression is rapidly downregulated after 4 SDs in the leaves of (E) WT, (F) LAP1oe-line 1A, and (G) LAP1oe-line 9A. The graphs indicate mean (±SE; three biological replicates) *FT2* transcript levels relative to those of the reference gene *UBQ* (y axis) versus duration in days of SD treatment (x axis).

See also Figure S4.

whether *LAP1* expression responds to SDs. Therefore, we analyzed *LAP1* expression in leaves (in which the SDs are perceived) and the apex (in which the growth cessation response is manifested). *LAP1* was expressed in the apex and, much more weakly, in the leaves (Figure 2A). We also generated transgenic hybrid aspen plants expressing the bacterial *uidA* (β-glucuronidase) reporter gene under the control of *LAP1* promoter. In agreement with the data from RT-PCR, the expression of the *uidA* reporter was detectable in the apex and vascular tissues of the transgenic *LAP1:uidA* plants (Figure S4A). We then investigated whether *LAP1* expression responds to SDs in the apex and observed reduced *LAP1* expression in the apex of the WT plants after just 2 weeks of exposure to SDs (Figure 2B). In contrast, no reduction in *LAP1* expression was observed in apices of WT plants grown for 2 weeks in long days (Figure S4B). Thus, *LAP1* is expressed in the apex, and its expression is downregulated by SDs.

LAP1 Acts Downstream of the CO/FT Module

We then tested the hypothesis that *LAP1* acts downstream of the CO/FT module by comparing the responses of *LAP1* expression to SDs in WT and in FToe plants in which SD response is defective [12]. In contrast to WT, we observed little, if any, downregulation of *LAP1* expression in response to SD treatment in FToe plants (Figure 2C). Moreover, consistent with our hypothesis, steady-state transcript levels of *LAP1* in the apex of FToe plants were significantly higher than those in the WT plants under long days, and, conversely, *LAP1* expression was lower in the FT-RNAi plants, which have reduced *FT* expression [12] and respond more rapidly to SDs (Figure 2D). Thus, these results suggested that *LAP1* could act downstream of the CO/FT module.

If *LAP1* acts downstream of the CO/FT module, the SD perception should function normally in LAP1oe plants despite attenuation of the growth cessation response. To address this, we compared the downregulation of *FT2* expression to SDs in the WT and LAP1oe plants. Downregulation of *FT2* by SDs is the earliest known measurable response to SDs [15, 16]. In the WT (Figure 2E) and in the LAP1oe plants (Figures 2F and 2G), *FT2* expression was downregulated in a similar manner after 4 SDs, indicating that LAP1oe plants are able to perceive SDs like the WT plants do. These data strongly suggest that *LAP1* is not involved in SD perception but acts downstream of the CO/FT module in the signaling pathway.

Defect in SD-Mediated Growth Cessation by Overexpression of *FT* Is Suppressed by Downregulation of *LAP1*

Overexpression of *FT1* or *FT2* has been previously shown to attenuate SD perception and leads to failure to cease growth in response to SDs [12–14]. In addition, FToe plants do not downregulate *LAP1* expression in response to SDs (Figure 2C). Therefore, we tested the hypothesis that the failure of *FT*-overexpressing plants to cease growth could be due to their inability to downregulate *LAP1* expression in SDs. To address this, we took a genetic approach by reducing the expression of *LAP1* in transgenic hybrid aspen plants that ectopically express FT1-GFP-hemagglutinin fusion (FT1-GFP-HAoe) (Figure S5). We compared the growth cessation response in FT1-GFP-HAoe (FT1 overexpressors) with FT1-GFP-HAoe/LAP1-RNAi lines (reduced *LAP1* expression in FT1 overexpressor background) after SD treatment (Figure 3). The FT1-GFP-HAoe plants continued to grow, formed new leaves, and did not form buds (Figure 3A), even after 10 weeks of SDs, whereas FT1-GFP-HAoe/LAP1-RNAi plants ceased growth, stopped making new leaves, and set buds when grown under similar conditions (Figures 3B–3D and S5B). Thus, the reduction of *LAP1* expression is sufficient to suppress the defect in SD-mediated growth cessation response resulting from the overexpression of *FT1*.

SD-Mediated Downregulation of *AIL1* Expression Is Modulated by *LAP1*

AIL1 is a target of SDs downstream of the CO/FT module, and downregulation of *AIL1* expression is required for growth cessation [16]. Moreover, *AIL1* overexpressors display severe

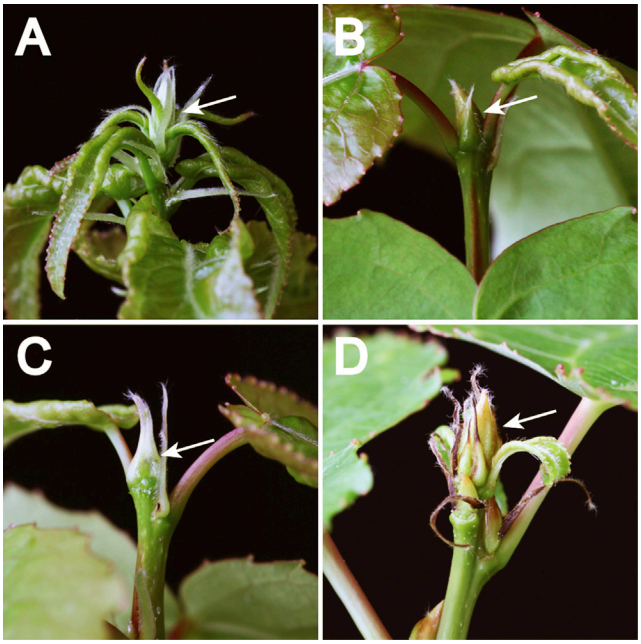

Figure 3. Downregulation of *LAP1* Expression Suppresses Growth Cessation Defect in *FT1*-Overexpressing Plants

Plants expressing FT1-GFP-HAoe and FT1-GFP-HAoe/LAP1-RNAi were subjected to SDs.
(A) FT1-GFP-HAoe plants did not cease growth, as manifested by actively growing apex (arrowed).
(B–D) In contrast, growth cessation and bud set (arrowed) were observed in three independent FT1-GFP-HAoe/LAP1-RNAi plants after SD treatment: (B) line 7, (C) line 8, and (D) line 10.
See also Figure S5.

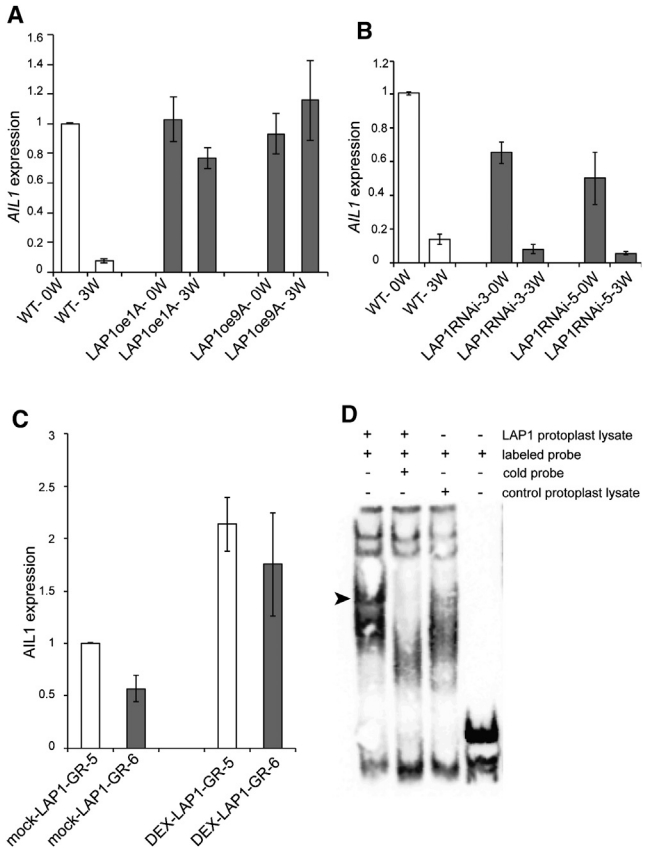

Figure 4. AIL1 Is the Downstream Target of LAP1

(A and B) Expression of *AIL1* in apices of WT and LAP1oe lines 1A and 9A (A) and in LAP1-RNAi lines 3 and 5 (B) after SD treatment. The graphs indicate mean (±SE; three biological replicates) expression levels of *AIL1* relative to those of the reference gene *UBQ* (y axis) versus duration of SD treatment in weeks (x axis).
(C) Induction of *AIL1* expression in apices of LAP1-GR-HA-expressing plants after dexamethasone treatment for 4 hr. The transgenic lines and treatments used are indicated on the x axis, and *AIL1* expression (average of three biological replicates ± SE) relative to reference gene *UBQ* is indicated on the y axis.
(D) HA-tagged LAP1 protein interacts with *AIL1* promoter (lane 1–4). Components of the reaction mixtures used in the EMSAs are indicated.
See also Figures S5C and S5D.

attenuation of growth cessation response in SDs similar to the way the LAP1oe plants do. Therefore, we investigated whether the regulation of *AIL1* by SDs is perturbed in LAP1oe and LAP1-RNAi plants. In agreement with earlier results (Figure 4A), *AIL1* expression was downregulated in the apices of the WT plants after 3 weeks in SDs. In contrast, no downregulation of *AIL1* expression was observed in LAP1oe plants (Figure 4A). Conversely, the expression of *AIL1* was lower in LAP1-RNAi plants than in the WT controls both before the start and after 3 weeks of SD treatment (Figure 4B).

LAP1 Induces *AIL1* Expression and Can Interact with the *AIL1* Promoter

Because the SD-mediated regulation of *AIL1* was attenuated in LAP1oe and LAP1-RNAi plants, we investigated whether *LAP1* induction can influence *AIL1* expression. For this, we generated transgenic hybrid aspen plants expressing a glucocorticoid receptor (GR) fusion of LAP1 (LAP1-GR-HA), permitting the LAP1-GR fusion to enter the nucleus and activate *LAP1* target genes, following the addition of dexamethasone. After treating the apices of LAP1-GR-HA-expressing plants for 4 hr, *AIL1* transcript levels were significantly higher than in the mock-treated controls, suggesting that *LAP1* can activate *AIL1* expression (Figure 4C).

Following this confirmation that *LAP1* can activate expression of *AIL1*, we investigated the ability of LAP1 to interact with the *AIL1* promoter by using electrophoretic mobility shift assays (EMSAs) that used cell extracts from *Arabidopsis* protoplasts expressing HA-tagged LAP1. When a 1.3 kb region of *AIL1* promoter upstream of the *Arabidopsis thaliana* genome (ATG) codon (divided into seven fragments of circa 150–200 base pairs [bp]) was investigated, a clear shift was observed only with a fragment (F1) of *AIL1* promoter close to the ATG codon when using extracts containing HA-tagged LAP1. In contrast, no shift was observed when other fragments (F2–F7) further upstream in the *AIL1* promoter were tested (Figure S5C). Furthermore, binding of HA-tagged LAP1 with *AIL1* promoter fragment F1 (Figure 4D) (lane 1) could be competed out with unlabeled specific competitor F1 (lane 2), whereas no shift was observed with the same promoter fragment when using control extracts from untransformed protoplasts (lane 3). Importantly, we also compared the ability of unlabeled specific fragment F1 and unspecific fragment F3 (from *AIL1* promoter) to compete with labeled F1 fragment in binding with HA-tagged LAP1 (Figure S5D). This assay showed that unlabeled fragment F1 could efficiently compete out the binding of labeled F1 with HA-tagged LAP1 at much lower concentration

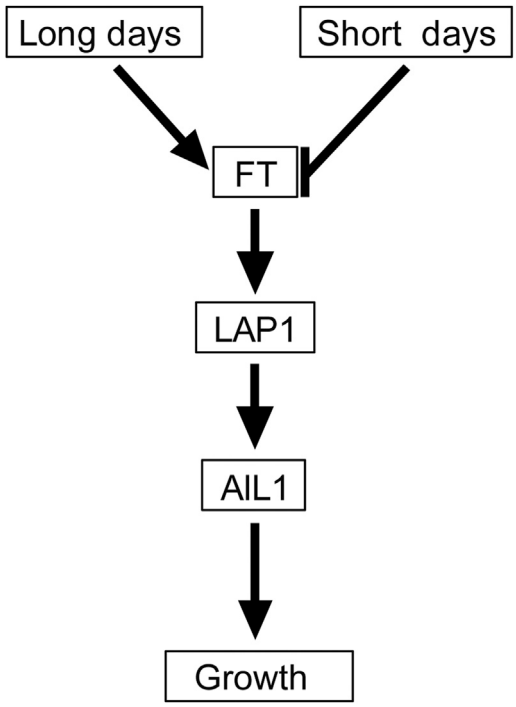

Figure 5. A Model for Photoperiodic Control of Seasonal Growth in Trees
See Discussion for details.

than unspecific fragment F3 could (Figure S5D). Whereas unlabeled F1 significantly reduced binding already at 50-fold excess and nearly abolished binding at 100-fold excess, significant binding of labeled F1 with HA-tagged LAP1 was observed even when 500-fold excess of fragment F3 was used in competition. Thus, a significantly lower concentration of specific competitor compared with unspecific competitor is required for reducing the binding of labeled specific fragment with HA-tagged LAP1. Taken together, these data support the hypothesis that *LAP1* is involved in the regulation of *AIL1* expression.

Discussion

Downregulation of *LAP1* Is Required for the SD-Mediated Growth Cessation

In the screen for factors involved in SD-mediated growth cessation, we identified *LAP1* as a target of SDs. In agreement with the hypothesis that *LAP1* downregulation by SDs is required for the induction of growth cessation, *LAP1* is expressed in the apex in which the growth cessation response is manifested, and *LAP1* expression is downregulated after exposure to SDs. Most importantly, SD-mediated growth cessation is severely compromised when *LAP1* expression cannot be downregulated after SD treatment like it can in FT-overexpressing or LAP1oe plants. Conversely, LAP1-RNAi plants with reduced *LAP1* expression cease growth early after SD treatment compared to the WT plants. These findings demonstrate that SD-mediated induction of growth cessation requires the downregulation of *LAP1* expression.

LAP1 Is a Target of SDS Downstream of the CO/FT Module

The downregulation of *FT2* after SD treatment is a key, established event in the induction of growth cessation [12], but little was previously known about factors acting downstream of the *CO/FT* module targeted by SDs. Our finding that LAP1oe plants have a similar phenotype to *FT* overexpressors (because they do not cease growth in response to SDs) prompted us to investigate whether *LAP1* acts in the same pathway as the *CO/FT* module, and, if so, whether it acts upstream or downstream. Several lines of evidence that we acquired indicate that *LAP1* acts downstream of the module. First, the SD-mediated downregulation of *LAP1* expression is attenuated in *FT*-overexpressing plants. Second, *FT* levels are positively correlated to *LAP1* expression. Third, *LAP1* overexpression does not affect SD perception, as demonstrated by the downregulation of *FT2* in response to SDs, in marked contrast to the attenuated expression of *LAP1* in *FT* overexpressors. Finally and most importantly, downregulation of *LAP1* expression is sufficient to suppress the growth cessation phenotype resulting from overexpression of *FT*. Taken together, these results indicate that *LAP1* is a target of SDs downstream of the CO/FT module.

LAP1 Connects the *CO/FT* Module with the Regulation of *AIL1*

The SD signal transduced via the CO/FT module targets the cell-proliferation regulator *AIL1*. Downregulation of *AIL1* expression is essential for the SD-mediated induction of growth cessation, and hybrid aspen plants overexpressing *AIL1* do not cease growth in response to SDs like LAP1oe plants do [16]. However, because the CO/FT module does not directly control the expression of *AIL1*, we postulated that some components acting between the CO/FT module and *AIL1* must be targeted by the SDs. Because *LAP1* acts downstream of the CO/FT module, an attractive hypothesis was that *LAP1* could mediate in the regulation of *AIL1* by the CO/FT module. Several lines of evidence support such a role of *LAP1*, including the observation that *AIL1* downregulation under SD treatment is severely attenuated in LAP1oe plants. Conversely, LAP1-RNAi plants have lower *AIL1* expression compared to the WT plants, both before and under SDs. Importantly, induction of *LAP1* can activate the expression of *AIL1*, and LAP1 can interact with the *AIL1* promoter. Taken together, our data strongly suggest that *LAP1* is the hitherto-unknown factor that connects the CO/FT module with the regulation of downstream factors such as *AIL1*.

A Model for the SD-Mediated Control of Growth Cessation

Based on our observations, we propose the following model for SD-mediated control of growth cessation response (Figure 5). In long days, high *FT* expression maintains *LAP1* expression above a threshold that allows continued growth. After a shift to short days, reduction in *FT* expression causes *LAP1* expression to fall below the threshold required for growth in the apex. This reduction of *LAP1* expression after SD treatment leads to a downregulation of *AIL1*. Because *AIL1* is a positive regulator of core cell-cycle genes such as D type cyclins, downregulation of *AIL1* expression would result in the downregulation of core cell-cycle genes, inducing growth cessation and bud set. Importantly, although our data identify *LAP1* as a target of SDs downstream of CO/FT whose downregulation is essential for SD-mediated growth cessation, we cannot exclude the possibility that there could be additional targets of SDs that act redundantly with *LAP1* and are also involved in the growth cessation response.

LAP1 May Function Primarily in Growth Cessation Rather Than Flowering

It has been suggested that the ancient function of *AP1*-like genes was in floral development [22] and that its function in vernalization (e.g., in cereals) [23] was acquired later, presumably by low-temperature-regulated expression of cereal *AP1* ortholog. Hybrid aspen *LAP1* not only displays high similarity to the *Arabidopsis* floral meristem identity gene *AP1* at the amino acid level but also functions like *AP1* when expressed in *Arabidopsis*. Intriguingly, in contrast with *AP1* overexpression in *Arabidopsis*, we did not observe early flowering in hybrid aspen *LAP1* overexpressors. The underlying reason for differences in function of *LAP1* in hybrid aspen and *Arabidopsis* is not entirely clear but could be due to differences in the downstream targets of *LAP1* in hybrid aspen (e.g., *AIL1*) compared with *Arabidopsis*. Similar results have also been observed when analyzing the function of hybrid aspen *FT* genes in *Arabidopsis* [12–14]. It is also possible that fundamental differences in flowering between trees (e.g., hybrid aspens [in which flowering occurs only after several years] and annual plants such as *Arabidopsis*) might be associated with differences in the function of *LAP1* in hybrid aspens compared with *Arabidopsis*. Nevertheless, despite the functional similarities between tree *LAP1* and *Arabidopsis AP1* and despite acting downstream of the CO/FT module, *LAP1* appears to primarily be involved in photoperiodic control of seasonal growth in hybrid aspen. Thus, our results implicating *LAP1* in photoperiodic control of seasonal growth reveal a novel role for *AP1*-related genes that has not been previously described.

Conclusions

Modulation of development by environmental signals plays a key role in the survival of plants and the adaptation of plants to seasonal changes. Photoperiod is a major environmental signal regulating a highly diverse developmental transition in plants. An important unresolved question is how has the signaling machinery evolved to mediate in photoperiodic control of responses as diverse as flowering, tuberization, and seasonal control of growth? Identification of the conserved CO/FT module mediating in photoperiodically controlled responses suggests that the signaling pathway must have diverged downstream of this module. *AP1*, a downstream target of the CO/FT module, has been primarily associated with the regulation of flowering. Therefore, it was likely that the divergence in the photoperiodic signaling pathway could have occurred directly after the CO/FT module, with *AP1* and related genes being the flowering-related downstream targets. Surprisingly, our results suggest that the photoperiodic signaling pathway could be conserved all the way to *AP1*, and that, instead, divergence could occur downstream of it. Identification of cell-proliferation regulator *AIL1* as a downstream target of *LAP1* reveals how a novel developmental process can now be controlled by an evolutionarily conserved signaling module in response to a photoperiodic input signal. Thus, these results provide insight into how the photoperiodic signaling pathway could have evolved to enable the regulation of diverse developmental pathways. In summary, our observations may suggest a general mechanism for the evolution of the photoperiodic signaling pathway involving divergence, potentially downstream of *AP1* rather than downstream of the CO/FT module. Finally, these results warrant analysis of *AP1*-like genes in photoperiodically controlled developmental transitions, such as tuberization in potatoes in which the downstream targets are currently not well characterized.

Experimental Procedures

Plant Material and Growth Conditions

Hybrid aspen (*Populus tremula* × *Populus tremuloides*) clone T89 (WT) and the transgenic plants were cultivated in half-strength Murashige and Skoog (MS) medium (Duchefa) under sterile conditions for 5 weeks and then transferred to soil and grown for another 4 weeks in the greenhouse (16 hr light, 22°C, and 66% relative humidity). For growth cessation analysis, the plants were shifted to growth chambers and initially grown under long days (LDs) (16 hr, 20°C light/8 hr, 15°C dark cycles) for 1 week for acclimatization and subsequently under SDs (8 hr, 20°C light/16 hr, 15°C dark cycles). Response to SDs was determined by monitoring bud set and counting the number of "new" leaves formed from the initiation of SD treatment to bud set; this provides a sensitive measure of SD perception and growth cessation responses because perception of SDs induces termination of new leaf formation [20]. For this purpose, the smallest leaf that was separated from the apex at the time of SD initiation was marked to distinguish it from those formed during the SD treatment.

Phylogenetic Analysis

Multiple sequence alignment was performed with ClustalW, and phylogenetic analysis was carried out with the neighbor-joining method by using the MEGA5 program [24]. The accession numbers of *Arabidopsis* AP1, CAL, FUL, AGL79, SEP3, SEP2, FLC, MAF5, SVP, and AP3 are AT1G69120.1, AT1G26310.1, AT5G60910.1, AT3G30260.1, AT1G24260.1, AT3G02310.1, AT5G10140.1, AT5G65080.1, AT2G22540.1, and AT3G54340.1, respectively. The transcript names of *Populus trichocarpa* genes PtAGL7 and PtAGL8.2 are Potri.004G115400 and Potri.008G098400, respectively.

Generation of Plasmid Constructs

Full-length *LAP1* cDNA was amplified by using cDNA prepared from mRNA that was extracted from hybrid aspen apex as template and the primers 5′-CACCATGGGAAGAGGTAGGGT-3′ and 5′-GGATCCTCATGCTCCGTAACCTC-3′. *LAP1* cDNA was cloned into the pENTR/D-TOPO donor vector (Invitrogen) and transferred into the pK2GW7 plant transformation vector to generate a plasmid-designated pK2GW7-LAP1, which was then transformed into agrobacterium GV3101pmp90RK [25]. In order to generate a LAP1-RNAi construct, a fragment of 208 bp was amplified by using primers 5′-CACCGAGTCTCAAAGAGCTA-3′ and 5′-ATCTTGCAGATCCCAGAATG-3′ and full-length *LAP1* cDNA as template. The amplified fragment was cloned into pENTR/D-TOPO (Invitrogen) and then transferred into the plant transformation vectors to generate LAP1-pK7GWIWG2 (I) and LAP1-PH7GWIWG2 (I) constructs. To generate a pKGW7-FT1-GFP-HA construct, an FT1-GFP fusion was amplified by using the primers 5′-CACCATGTCAAGGGACAGAGATCCTCTGAG-3′and 5′-CTATCCTGCATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTACTTGTACAGCTCGTCCATGC-3′, and the amplified fragment was cloned in pENTR/D-TOPO (Invitrogen) and then transferred into the plant transformation vector pK2GW7. To generate a LAP1-GR-HA construct, *LAP1* cDNA was amplified and cloned in pENTR/D-TOPO. The coding region of rat GR-hormone-binding domain fused with a HA tag was amplified by using primers 5′-AAAAAGGATCCGAAGCTCGAAAAACAAAG-3′ and 5′-AAAAAGGATCCCTATCCTGCATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTATTTTTGATGAAAC-3′, and then the GR-HA fragment was ligated into the BamHI site at the 3′end of the LAP1-D-TOPO plasmid to generate a LAP1-GR-HA fragment, which was then transferred into the plasmid pK2GW7. A 2-kb pair fragment from the region upstream of the ATG codon of the *LAP1* coding sequence was amplified by using the primers 5′-CACCCATATGCAACATTATGAATCAAT-3′ and 5′-CTCTCTCTCTCTCTCTCTCTCTCTAA-3′ and hybrid aspen genomic DNA as template, cloned into the pENTR/D-TOPO vector (Invitrogen), and then transferred into the pKGWFS7 binary vector to generate a LAP1-GUS plasmid [26].

Plant Transformation

Hybrid aspen was transformed as previously described [27]. The vectors pK2GW7-LAP1 and LAP1-PK7GWIWG2 (I) were used to transform WT plants to generate *LAP1*-overexpressing and -RNAi lines, respectively. FT1-GFP-HA-overexpressing plants (FT1-GFP-HAoe) were generated by using the plasmid pK2GW7-FT1-GFP-HA to transform WT plants. LAP1-PH7GWIWG2 (I) was transformed into FT1-GFP-HAoe plants to generate LAP1-RNAi plants with an FT1-overexpressing background (FT1-GFP-HAoe/LAP1-RNAi). The LAP1-GR-expressing hybrid aspen plants were

generated by using the plasmid pK2GW7-LAP1-GR-HA in transformation of hybrid aspen. The *LAP1*:*uidA* construct was transformed into WT plants to generate hybrid aspen plants expressing the *uidA* reporter under control of the *LAP1* promoter. WT *Arabidopsis thaliana* Col-0 was transformed by using the floral dip method [28] with pK2GW7-LAP1 to express hybrid aspen *LAP1* cDNA.

Analysis of *LAP1*-Expressing Col-0 Plants

WT *Arabidopsis thaliana* Col-0 and homozygous *LAP1*-expressing plants grown in long days (16 hr light, 22°C) were monitored, and both their flowering time (expressed as the total number of leaves produced before flowering) and floral morphology were recorded.

LAP1 Promoter Analysis

Apices from greenhouse-grown transgenic hybrid aspen plants expressing the *uidA* reporter gene were collected and incubated for 15 hr at 37°C in a solution containing 1 mM X-gluc, 1 mM $K_3Fe(CN)_6$, 1 mm $K_4Fe(CN)_6$, 50 mM sodium phosphate buffer (pH 7.0), and 0.1% (v/v) Triton X-100. The samples were then rinsed with water and visualized under a Zeiss Axioplan light microscope, and the images were analyzed by using Axiovision version 4.5 software (Zeiss).

RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was extracted from samples of tissues taken at the same time of day by using an Aurum Total RNA kit (Bio-Rad) or RNeasy Plus Universal Kit (QIAGEN). Portions (10 μg) of total RNA were treated with RNase-Free DNase (QIAGEN) and cleaned using an RNeasy Mini Kit (QIAGEN). We used 1 μg of the RNA from each sample to generate cDNA by using an iScript cDNA synthesis kit (BioRad). Selected (UBQ/TIP41-like) reference genes were validated using GeNorm Software [29]. Quantitative real-time PCR analyses were carried out with a Roche LightCycler 480 II instrument, and relative expression values were calculated by using the Δ-ct-method as previously described [16]. A complete list of primers used in real-time PCR analysis is presented in Table S1.

Generation of HA-Tagged LAP1 and Expression in *Arabidopsis* Protoplasts

LAP1 cDNA was amplified by using the primers 5′-AAAAAGGATCCATGG GAAGAGGTAGGGT-3′ and 5′-AAAAAAGGATCCTCATGCTCCGTAACCTC-3′, and the resulting fragment was cloned into pRT104-3xHA and used for the transfection of *Arabidopsis* protoplasts as described by [30], using 10 μg of purified plasmid. Cells were lysed in a buffer containing 25 mM Tris-HCL (pH 7.5), 50 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% Igepal, and 1X protease inhibitor cocktail (PIC). After centrifugation, the supernatant was collected and immediately frozen in liquid nitrogen. Expression of the HA-tagged LAP1 protein was confirmed by western blot analysis, and the resulting cell extracts were used for further analysis.

Generation of Labeled *AIL1* Promoter Fragments and EMSA

A total of seven (150–200 bp) fragments (F1–F7) of the *AIL1* promoter covering 1.3 kb of the region upstream of ATG were amplified by using biotin-labeled primers specified in Table S1 and *P. trichocarpa* genomic DNA. The fragments were purified by using an E.Z.N.A. Gel Purification Kit (Omega) followed by phenol-chloroform extraction and ethanol precipitation prior to use in gel-shift assays. EMSAs were performed using biotin-labeled promoter fragments and cell extracts from protoplasts expressing HA-LAP1 or control extracts from nontransformed protoplasts as previously described [16]. Briefly, 10 μl protoplast cell extract was mixed with 0.5 μl biotin-labeled DNA (10 fmol/μl), 0.4 μl nonspecific competitor (poly [dI:dC], 1 mg/ml), and 0.5 μl BSA (20 mg/ml) in EMSA buffer [16]. For specific competition, 500 fmol (100-fold excess) nonlabeled promoter fragment F1 was added to the reaction. For comparing the effect of specific and unspecific DNA on LAP1 binding with fragment F1, either excess unlabeled (cold) specific fragment F1 (25-, 50-, and 100-fold excess corresponding to 125, 250, and 500 fmol, respectively) or unlabeled (cold) unspecific fragment F3 (100-, 200-, and 500-fold excess corresponding to 500, 1,000, and 2,500 fmol, respectively) was added in addition to 5 fmol labeled F1 fragment in EMSAs. The mixtures were incubated on ice for 10 min followed by 30 min at room temperature to allow binding, and then the samples were electrophoretically separated in a nondenaturing polyacrylamide gel (5%) prepared in 0.5× TBE and transferred to a Hybond N+ membrane (GE Healthcare). Finally, a LightShift Chemiluminescent EMSA kit (Pierce) was used for crosslinking and detection.

Dexamethasone Induction Treatment

Apices of plants of two independent transgenic lines expressing the LAP1-GR-HA fusion grown in half-strength MS medium were incubated in a solution containing 10 μM dexamethasone (DEX) and 0.015% Silwet L-77 or 0.015% Silwet L-77 alone (mock for controls) for 4 hr. Following the treatment, the tissue was frozen until further use for mRNA isolation [31].

Supplemental Information

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.02.037.

Acknowledgments

We would like to thank Urs Fischer, Ove Nilsson, and Anna Karlberg for valuable suggestions and Laszlo Bako for advice on EMSAs. This work was funded by grants from SLU (Excellence), VR (Berzelii), and Formas (TC4F, Future Trees, Bioimprove) to R.P.B.

Received: December 11, 2013
Revised: February 6, 2014
Accepted: February 13, 2014
Published: March 20, 2014

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